Developmental Aspects of Xenobiotic Transformation

by W. Klinger* and D. Muller*

In most laboratory animals monooxygenases are apparently absent or barely detectable in fetal organs until just before birth. In this contribution hepatic cytochrome P-450-dependent reactions in the rat are considered only. The results are interpreted on basis of the reaction scheme of Estabrook. To avoid methodological pitfalls the basic kinetics for all reactions investigated have been investigated with liver preparations from newborn and adult rats. The low monooxygenase activity of rat liver during the perinatal period can be observed even under optimal conditions for the *in vitro* enzyme assay. There are different developmental patterns for various reactions O-demethylation of codeine, phenazone-hydroxylation, first and second steps of N-demethylation of amidopyrine, N-demethylation of ethylmorphine. There are marked differences not only in V_{max} but also in the postnatal development of k_m and the inducibility by phenobarbital. Thus the existence of a different cytochrome P-450 is evident also by this approach. The low monooxygenase activity of rat liver during the perinatal period is not due to a lack of NADPH or NADH, to an age-dependent NADPH cytochrome P-450 reductase activity or to an age-dependent NADH-cytochrome P-450 reduction. Moreover this low activity is not due to an insufficient mitochondria-endoplasmic reticulum interaction. It is accompanied by low ΔA_{max} after addition of a typical type I substrate (hexobarbital) and by a small amount of metyrapone-binding centers: it can be explained by a smaller percentage of active cytochrome P-450 in comparison to adult rat liver.

Since the first publications by Jondorg, Maickel, and Brodie (1) and Fouts and Adamson (2), several hundred papers have been published concerning the time of appearance and the rates of development of various microsomal mixed function oxidases, now named monooxygenases, in various mammalian species and different organs and tissues. Moreover, quite a lot of investigations were undertaken to test whether liver microsomal enzymes of fetuses or newborns were induced by treatment of the pregnant female at various stages of gestation, in order to attain either an earlier appearance or a more rapid enhancement of enzyme activity in fetal life.

In summary, these many investigations have shown that in most laboratory animals the monooxygenases are apparently absent or barely detectable in fetal organs, especially in the fetal liver, until just before birth.

Furthermore in most studies treatment of pregnant animals with enzyme inducers prior to the last few days of gestation had no effect on fetal hepatic drug-metabolizing enzyme activities. In most mammals and tissues the different biotransformation reactions develop after birth, but the developmental patterns differ widely. It has been pointed out that the balance between toxification and detoxification systems in various organs shifts considerably in the perinatal period (3), but one type of biotransformation reaction may mean a toxification for one substrate, for another one a detoxification. Therefore we shall prefer the neutral term "biotransformation" only (4-6). Moreover, we shall consider the liver parenchymal cells only. It is well known that there is age dependence of uptake into the cell, both by phase I reactions, which form polar

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metabolites capable of conjugation and of phase II reactions, i.e., formation of glucuronides and sulfates, and also of excretion out of the cell.

We shall consider here only phase I reactions and among these only those dependent on cytochrome P-450 (cyt. P-450). Phase II reactions and their perinatal development are discussed by Lucier (7). A synopsis of the reactions involved is given in Figure 1. For the explanation of the results reflected here the reaction scheme of Estabrook (8) may serve as our base (Fig. 2). The reaction sequence proposed in Figure 2 may be briefly described as follows: (1) an organic

substrate (S) reacts with the low spin form of the ferric hemoprotein to form a high-spin ferric substrate complex; (2) the ferric substrate complex undergoes a one-electron reduction to a ferrous substrate complex; (3) the ferrous substrate complex can react with carbon monoxide to form the familiar ferrous-carbon monoxide complex typified by the characteristic absorbance band at 450 nm, or with oxygen to form a ternary complex of heme iron, oxygen, and substrate; (4) The ferrous substrate-oxygen complex then undergoes a second stage of reduction; (5) the cycle is completed by insertion of one atom of the bound molecular oxygen into the organic substrate concomitant with the formation of water and splitting off the hydroxylated substrate. The overall reaction, explained by this scheme, reveals rather different developmental patterns for different substrates. I want to summarize only the results of our small group.

Results and Discussion

We investigated various model reactions for the cytochrome P-450-dependent microsomal monooxygenase: N-demethylation of amidopyrin; first and second demethylation step; O-demethylation of codeine hydroxylation of phenazone; reduction of p-nitrobenzoic acid (in microsomes and cytosol).

A review of the postnatal development of V_{max} is given in Figure 3 (9). On investigating the N-demethylation of amidopyrine in more detail, we found different developmental patterns for of the first and second step of N-demethylation, and
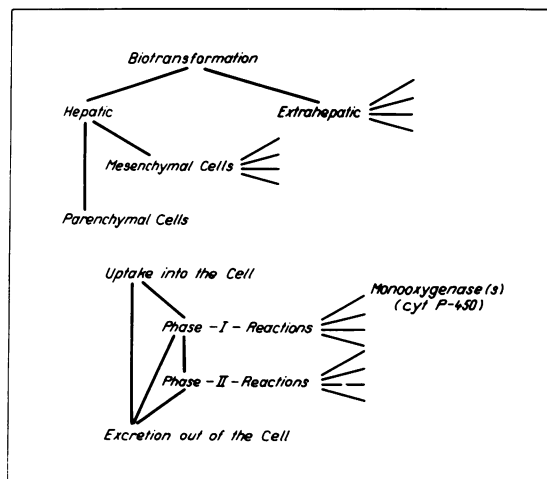

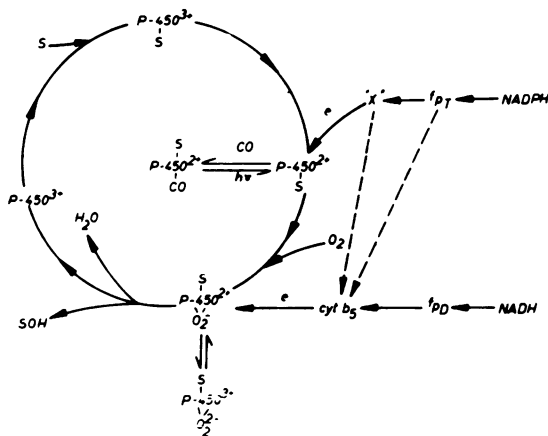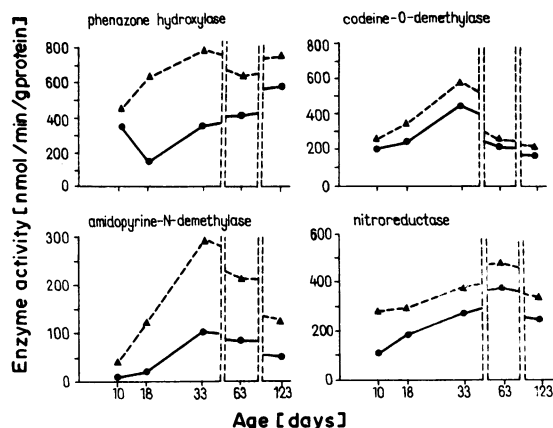FIGURE 1. Synopsis of biotransformation localization and steps.

FIGURE 2. Proposed scheme for the cyclic reduction and oxidation of cytochrome P-450 including a function for cyt b_5. The substrate to be hydroxylated is indicated by S. The flavoproteins NADPH-cyt P-450- and NADH-cyt b_5- reductase are indicated by f_{pT} and f_{pD}, respectively. X is a hypothetic mobile carrier, shuttling from a single flavoprotein dehydrogenase to various cyt P-450 molecules.

FIGURE 3. Developmental changes of activities of phenazone hydroxylase, amidopyrine-N-demethylase, codeine-O-demethylase, and nitroreductase in 9000 g liver supernatant of NaCl and barbital-treated (150 mg/kg) male rats: (●) controls; (▲) barbital-treated rats.

the following postnatal development. Figures 4 and 5 give the Michaelis-Menten plots of both demethylation steps: Lineweaver-Burk plots are given in Figures 6 and 7 (10,11). As early as 1969, at a symposium on microsomes in Tübingen we demonstrated these nonlinear kinetics and distinct changes during postnatal development, with differences for both demethylation steps.

For both demethylation steps we found different age-dependent phenobarbital inducibilities and inhibition effects different in inhibition type and rate, with phenylbutazone and phenobarbital as inhibitors *in vitro* (11).

We therefore switched to a less complicated model, the N-demethylation of ethylmorphine. We investigated this reaction with liver slices, homogenate, 9000 and 15000g supernatant, and with microsomes. To avoid methodological pitfalls, we investigated the basic kinetics with liver preparations of newborn and adult rats. The results with homogenate are as follows. The reaction followed a linear course up to 20 min and depended strongly on protein concentration of the homogenate with final dilution ranges of 1:36–1:600.
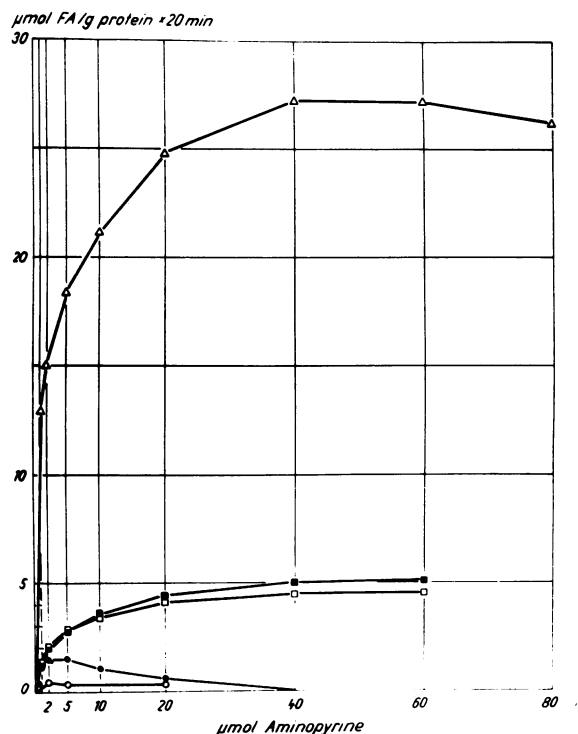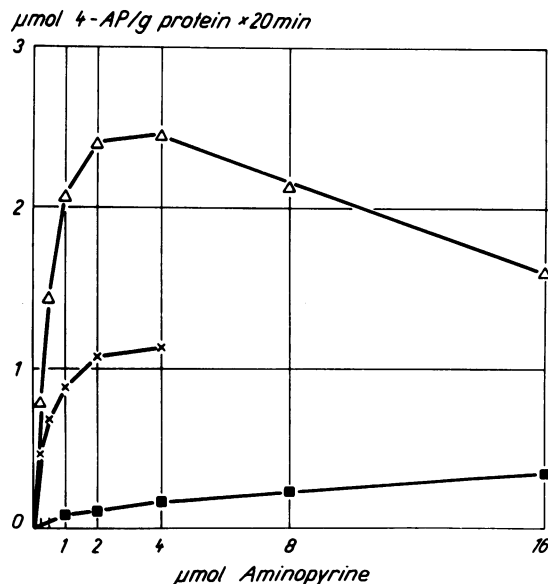
μmol 4-AP/g protein ×20min

FIGURE 5. Kinetics of the second step of amidopyrine N-demethylation by 9000 g liver supernatant of (■) liver of 10-day-old rats; (×) liver of 30-day-old rats; (△) liver of 120-day-old rats.

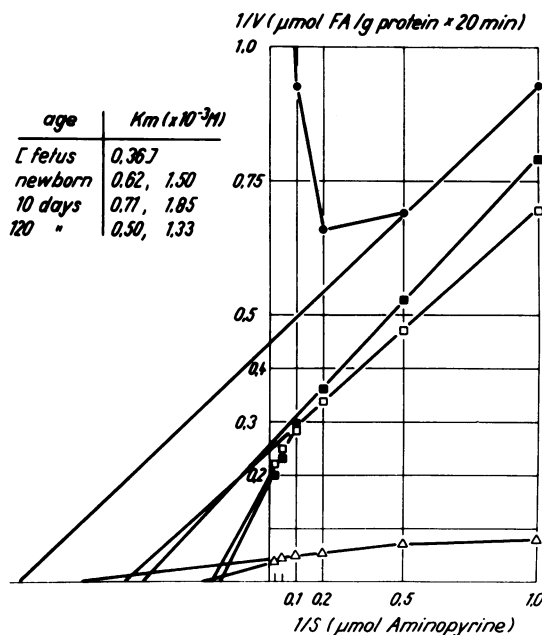1/V (μmol FA/g protein × 20 min)

age	Km (×10⁻³ M)
[fetus	0,36]
newborn	0,62, 1,50
10 days	0,71, 1,85
120 "	0,50, 1,33

1/S (μmol Aminopyrine)

FIGURE 6. Lineweaver-Burk plot to Fig. 4.

μmol FA/g protein ×20min

FIGURE 4. Kinetics of the first step of amidopyrine N-demethylation by 9000 g liver supernatant of (O) fetal liver, 15th day of gestation; (●) fetal liver 21st day of gestation; (□) liver of newborn rats; (■) liver of 10-day-old rats; (△) liver of 120-day-old rats.

The effects of concentrations of glucose-6-phosphate, NADP, nicotinamide, and semicarbazide as well as of the molarity of the buffer used and the pH are shown in Figures 8 and 9. In both age groups, the reaction is glucose-6-phosphate- and NADP-dependent. To obtain max-
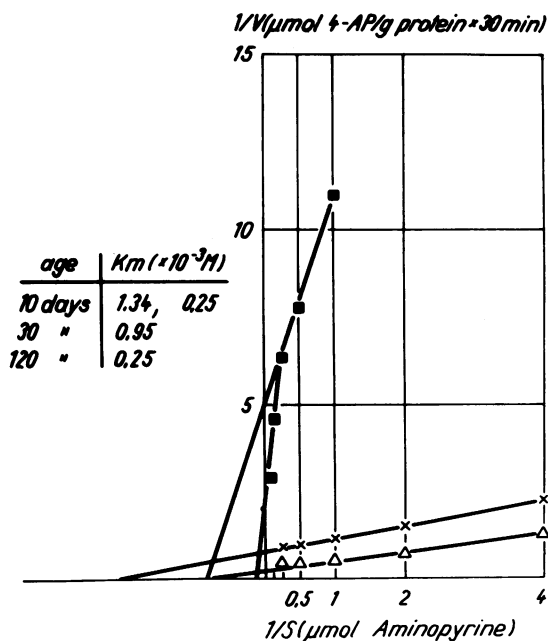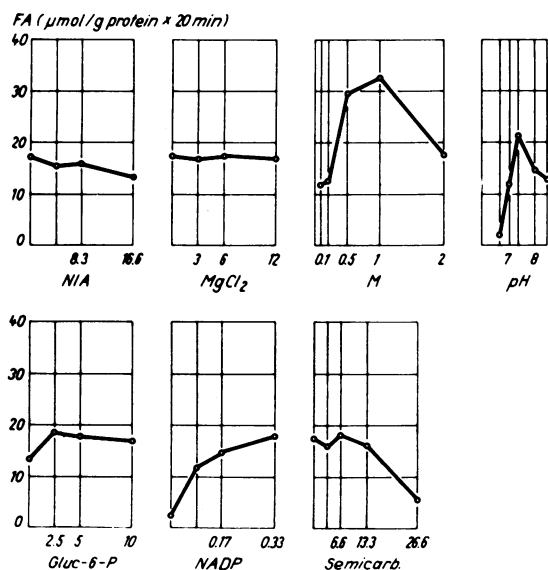
FIGURE 7. Lineweaver-Burk plot to Fig. 5.

FIGURE 8. Influence of nicotinamide (NIA), MgCl₂, molarity (M), pH, gluc-6-P, NADP, and semicarbazide on N-demethylation ethylmorphine by liver homogenate of newborn rats.

FIGURE 9. Infulence of nicotinamide (NIA), MgCl₂, molarity (M), pH, gluc-6-P, NADP and semicarbazide on N-demethylation of ethylmorphine by liver homogenate of adult rats.

imum activity, liver homogenate of newborn rats must be supported by higher concentrations of NADP than adult liver homogenate. Nicotinamide and MgCl₂ had no influence 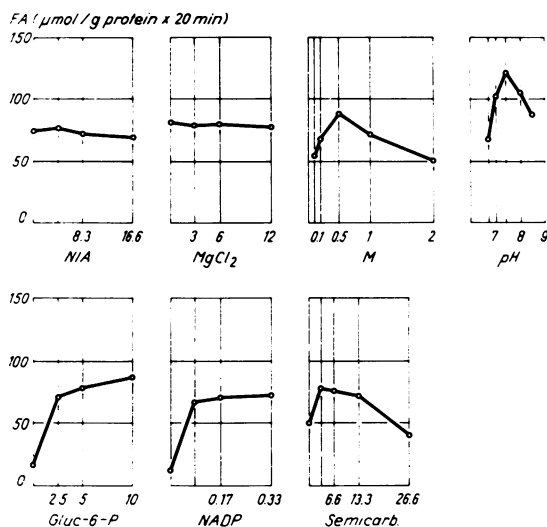on the reaction with liver homogenate from either newborn or adults rats. We then omitted the usual ingredients nicotinamide and MgCl₂. The optimal buffer molarity was 0.5M with newborn liver homogenate and 1.0M with adult liver homogenate. Trapping of the released FA with semicarbazide is not necessary with newborn liver homogenate, but it is required for adult liver homogenate, which has much higher activity. With both age groups the optimum pH was 7.4. Moreover there were differences between adult and newborn rat liver when the homogenates are stored. The results are shown in Figure 10.

Surprisingly, homogenate of newborn rat liver increases its activity when it is stored at −20°C and thawed immediately before use. Homogenate derived from adult rat liver is rather stable for at least 9 days; no activation was found. With liver homogenate, the course of V_{max} is demonstrated in Figure 11.

After the initial postnatal increase, the activity declines between the first and second week of age, and thereafter increases again to reach a maximum at 60 days. We thus confirmed and extended the findings of various groups (12-17), especially the decrease of activity in the second week of age. This decrease is true for succinate dehydrogenase and mitochondrial GOT, also (Barth and Klinger, unpublished data, 18). So we checked whether this pattern reflects an age-dependent mitochondria-endoplasmic reticulum interaction (19). In contradiction to the results of Cinti et al. (20), we could not find an effect of the
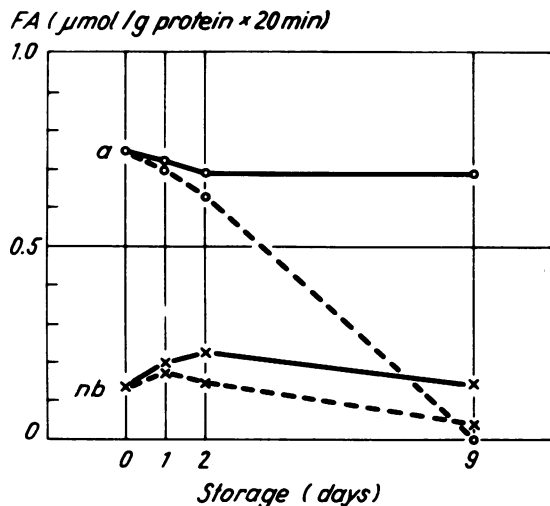
FA (μmol/g protein × 20min)

FIGURE 10. Influence of storage of liver homogenate of (×) newborn and (○) adult rats (—) at -20°C and (--) at +4°C.
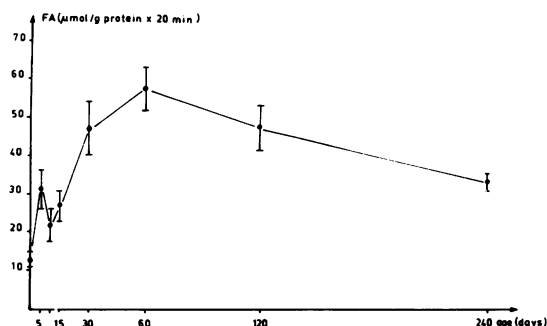

FIGURE 11. Age course of V_{max} of N-demethylation of ethylmorphine. Given are arithmetic means ± S.E.M., $n = 6$.

Krebs cycle intemediates in adults, nor could we demonstrate a significant influence on liver preparations with newborn liver. Figures 12 and 13 show the results with liver slices.

In conclusion, a lack of positive mitochondria-endoplasmic reticulum interaction is very probably not the reason for the low monooxygenase activity in newborn liver.

The determination of the K_m values could give a hint whether the postnatal development is associated with qualitative changes of any part of the microsomal electron transport chain. We plotted the values obtained with homogenated by Lineweaver-Burk, plot, and Endrenyi and Kwong plots (21) (Figs. 14 and 15). Only with liver homogenate of newborn animals did we obtain

straight lines and a single apparent K_m value: 0.34 mmole/l. With older age groups we found nonlinear kinetics and additional apparent K_m values. When the experimental data are plotted according to Endrenyi and Kwong, the graphs seem to be nearly linear, but this impression depends on the measure of the scale. Without exception, all graphs with adult rats were nonlinear. We repeated the determination of the apparent K_m values with adult rat liver homogenate more than ten times, to demonstrate the significance of this finding: In all cases the angle in the graphs could be reproduced. Qualitatively the finding is significant, as the angles always have the same characteristics.

The observed shape of the Lineweaver-Burk plot is qualitatively compatible with the assumption of two enzymes with different V (V_{max}) and K (K_m). If only negligible amounts of effectors are bound to both of them, then

$$V = \frac{V_1 S}{K_1 + S} + \frac{V_2 S}{K_2 + S} \qquad (1)$$

$$\frac{1}{V} = \frac{K_1 K + (K_1 + K_2)S + S^2}{(V_1 K_2 + V_2 K_1)S + (V_1 + V_2)S^2} \qquad (2)$$

$$\frac{1}{V} = \frac{a}{b}\left(\frac{1}{S}\right) + \frac{c}{b^2} - \frac{d}{b^2(b/S + e)} \qquad (3)$$

where

$a = K_1 K_2$
$b = V_1 K_2 + V_2 K_1$
$c = V_1 K_1^2 + V_2 K_1^2$
$d = V_1 K_2 (K_1 - K_2)^2$
$e = V_1 + V_2$

The first two right-hand terms of Eq. (3) correspond to a regular doubly reciprocal plot, whereas the third term produces a decrease of $1/v$ which tends to zero if $1/S = \infty$. Hence the plot is concave downward in accordance to the experiments. Thus it was tried to determine V_1, K_1, V_2, K_2 by fitting Eq. (1) to the measured data. The results show, however, that no unique evidence for the action of more than one enzyme can be gained in this way from the data. As may be seen from Figure 16, the curves fitted to Eq. (1) and to $v = VS/(K + S)$ differ only insignificantly from each other with regard to the scatter of the experimental data. Applying Fisher's test to the reduction of the lack of fit as proposed by Petterson and Petterson (22) also gives only an insignifi-
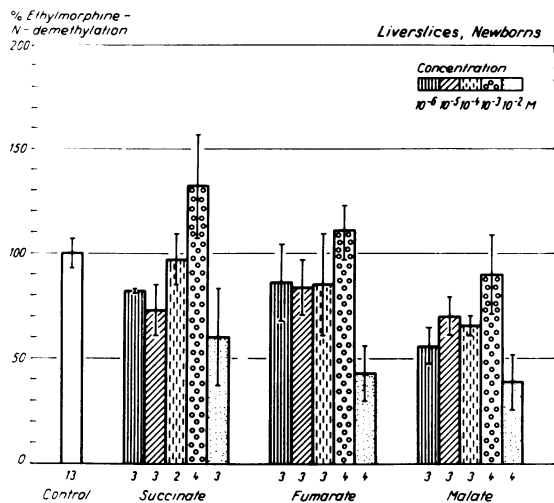
FIGURE 12. Influence of succinate, fumarate, and maleate at various concentrations (10^{-6}-$10^{-2}M$) on N-demethylation of ethylmorphine by liver slices of newborn rats. Given are arithmetic means ± S.E.M.; n is noted under the abscissa. For each value the livers of three animals were pooled. Demonstrated are relative changes; controls = 100%.
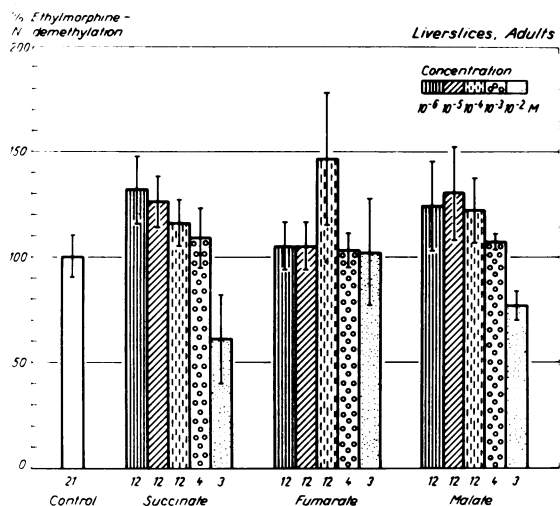

FIGURE 13. Influence of succinate, funarate, and maleate at various concentrations (10^{-6}-$10^{-2}M$) on N-demethylation of ethylmorphine by liver slices of adult rats. Given are arithmetic means ±1 S.E.M.; n is noted under the abscissa. Demonstrated are relative changes, controls = 100%.
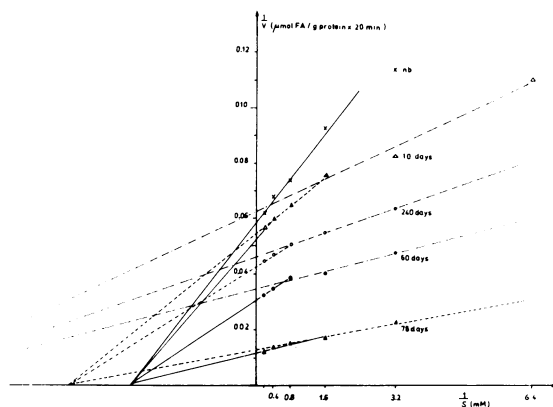

FIGURE 14. Lineweaver-Burk plot of N-demethylation of ethylmorphine by liver homogenate of male rats of various ages.

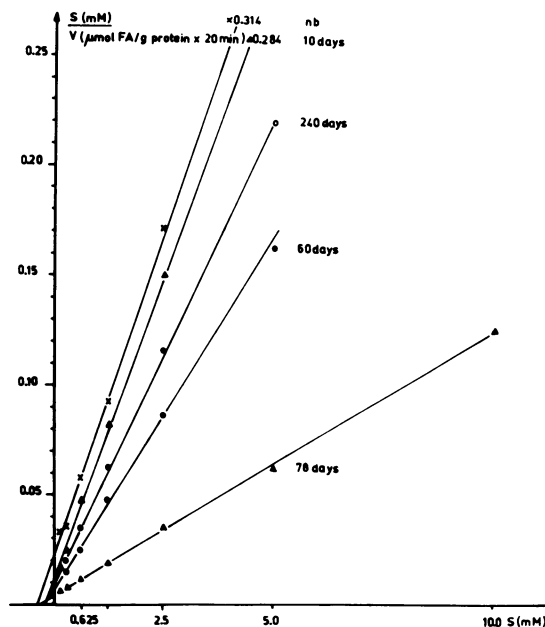FIGURE 15. Endrenyi-Kwong plot of same values given in Fig. 14.

cant improvement on assuming two enzymes. Further investigations are now in preparation. Phenobarbital pretreatment enhances not only V_{max}, but also changes the Lineweaver-Burk plot. The typical angle with control rats is abolished, and only one K_m value is obtained (Fig. 17).

After a thorough investigation of the basic kinetics and the confirmation of the course with age we are quite sure, that there is a postnatal development and that there are not only simple quantitative changes, but also qualitative ones. As no remarkable age differences in microsomal
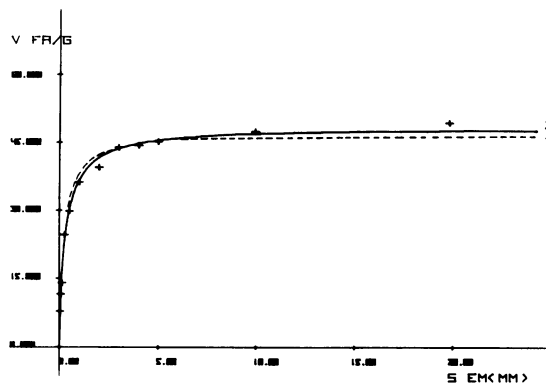
FIGURE 16. Michaelis-Menten plot of the N-demethylation of ethylmorphine by liver homogenate of adult rats (mean of three experiments, each with pooled liver homogenate of three rats). The graph was fitted by a Hewlett-Packard-calculator HP 20: (1) according to the one-enzyme model; (2) according to the two-enzyme model.
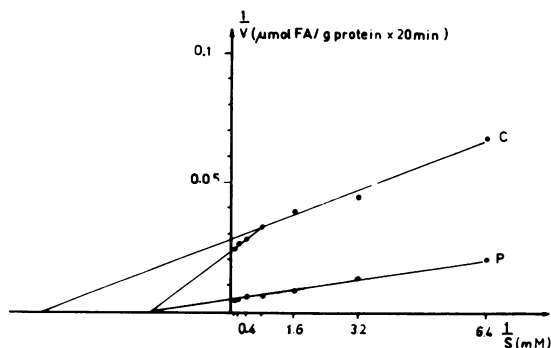

FIGURE 17. Lineweaver-Burk plot of N-demethylation of ethylmorphine by liver homogenate of adult male rats: (C) NaCl or (P) phenobarbital-pretreated $(3 \times 80 \text{ mg/kg})$ rats.

SH-group concentration and lipid peroxidation could be detected, we started to investigate the cytochrome P-450 cycle step by step, according to the Estabrook scheme: (a) spectral changes due to the binding of type I and type II substrates, (b) the amount of accessible cytochrome P-450 for the binding of substrates; (c) the first reduction step (NADPH-cytochrome P-reductase); (d) the second reduction step (provided by NADH).

Spectral Changes. The spectral changes due to the binding of hexobarbital (type I substrate) and aniline (type II substrate) were investigated (23). The K_s values for both substances and consequently the affinity for cytochrome P-450 do not change during aging. Phenobarbital does not alter the affinity of hexobarbital, but enhances the K_s value for aniline.

The maximal spectral changes ΔA_{max} due to aniline are nearly equal in all age groups;

whereas those for hexobarbital are small in young rats and increase considerably during aging. The age dependence of the hexobarbital-induced ΔA_{max} is similar to the development of drug-metabolizing reactions, (Figs. 18 and 19). The reason for the age dependence of type I spectral changes could be formation of different amounts of cytochrome P-450-substrate complex; in young animals the portion of cytochrome P-450 which interacts with hexobarbital is smaller than in adults, in spite of about the same cytochrome P-450 content. It is well known that not all cytochrome P-450 must be active; even in microsomes of adult rats the type I substrate cyclohexane converts only 12% of cytochrome P-450 to the substrate-complex (24). We therefore attempted to find another method to check this hypothesis.

Titration of Active Cytochrome P-450 by Metyrapone. According to Werringloer and Estabrook (25), not all cytochrome P-450 can bind metyrapone; this metyrapone-reactive cytochrome P-450 is enhanced by phenobarbital pretreatment. Roots and Hildebrandt $(26,27)$ demonstrated that the increase in metyrapone-binding sites by phenobarbital pretreatment corresponds to the increase in drug metabolism.
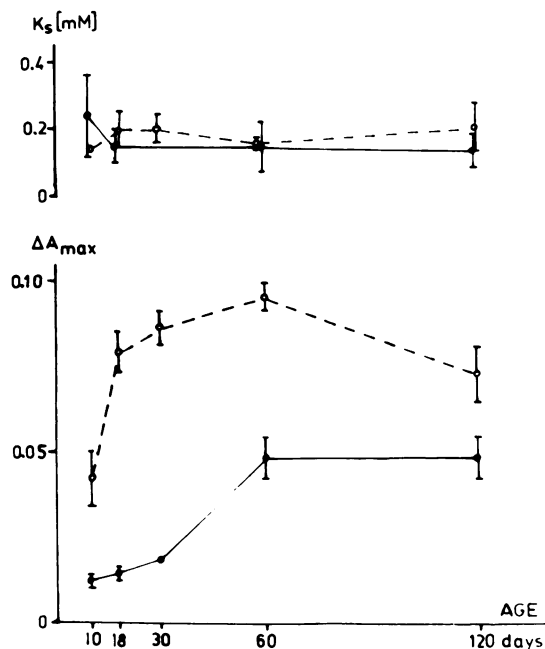

FIGURE 18. Spectral dissociation constant (K_s) for hexobarbital and maximum spectral changes (ΔA_{max}) caused by addition of hexobarbital with liver microsomes of rats of different ages: (●) control rats; (○) phenobarbital-pretreated $(3 \times 60 \text{ mg/kg})$ rats.
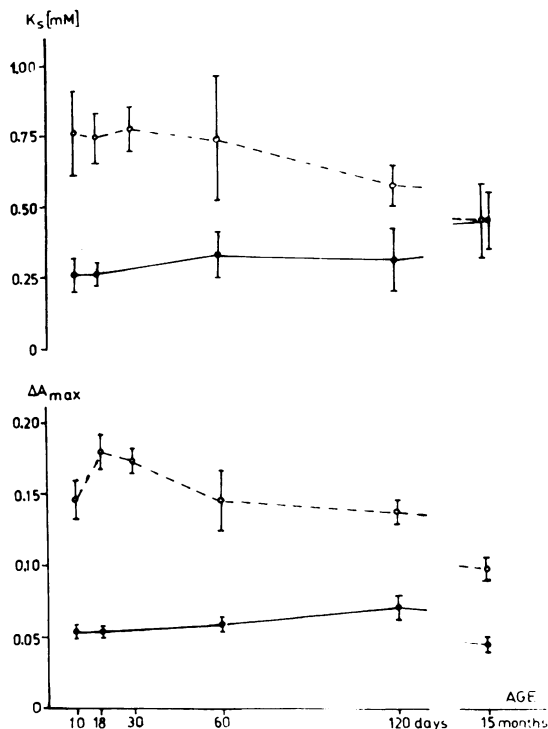
FIGURE 19. Spectral dissociation constant (K_s) for aniline and maximum spectral changes A_{max} caused by addition of aniline with liver microsomes of rats of different ages: (●) control rats; (○) phenobarbital-pretreated (3 × 60 mg/kg) rats.

FIGURE 20. Dependence of IC_{50} of metyrapone on protein concentration (a) in 18-day-old and (b) 60-day-old rats.

They concluded that phenobarbital enhances drug metabolism by increasing the number of active centers of cytochrome P-450 and not by increasing the turnover rate of cytochrome P-450. We determined the amount of metyrapone-reactive binding sites in liver microsomes from 18- and 60-day-old rats. In very low concentrations (1-4 μmole/l.), metyrapone inhibits N-demethylation of ethylmorphine noncompetitively; enzyme and inhibitor are present in the same order of magnitude. With this mutual depletion system (28) a determination of binding sites is possible. The following formula was used; $IC_{50} = K_i + E_t$ (where E_t is the amount of enzyme that binds metyrapone). IC_{50} was determined with the aid of a Dixon plot. Different protein and metyrapone concentrations and constant ethylmorphine concentration (4 mmole/l.) were used. Figure 20 shows the dependence of IC_{50} on protein concentration. Microsomes from 18- and 60-day-old rats were used. Since the cytochrome P-450 content per milligram protein is similar in both age groups, the protein concentration is a measure of the cytochrome P-450 concentration.
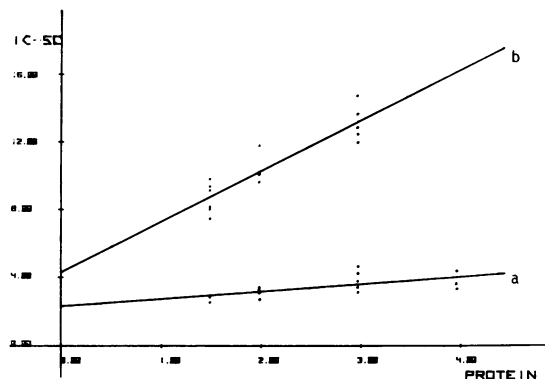
Figure 20 indicates that microsomes from adult rats bind more metyrapone than those from young rats. That means that the portion of metyrapone-reactive cytochrome P-450 is greater in adult than young animals. Figure 21 shows the dependence of IC_{50} on V_{max} for N-demethylation of ethyl-morphine. The straight lines for both age groups are nearly parallel, i.e., the correlation between V_{max} and E_t is the same in young and adult rats.

These findings support our assumption that the age dependence of N-demethylation of ethylmorphine is due to different portions of active cytochrome P-450 in spite of nearly the same total cytochrome P-450 concentrations. The metyrapone binding seems to be a measure of this active cytochrome.

First Reduction Step. As demonstrated in Figure 22, with adult liver homogenate no saturation could be achieved with NADPH, while with newborn liver homogenate the concentration of 1 mmole/l. is optimal. NADH alone without NADPH or NADPH regenerating system results in very low activities.

Previous investigations on the age dependence of NADPH-cytochrome P-450-reductase were carried out without adding type I substrates. No significant age differences were found with rabbit liver microsomes (29), whereas the rate increases during aging in liver microsomes of rats and pigs. We investigated the activity of NADPH-cytochrome P-450-reductase in rat liver microsomes with and without addition of type I substances in dependence on age after pretreatment with phenobarbital (30). Since the reduction was of first order only in the first 6-8 sec, this interval was used for determination of the half-life. From the half-life and the amount of cytochrome
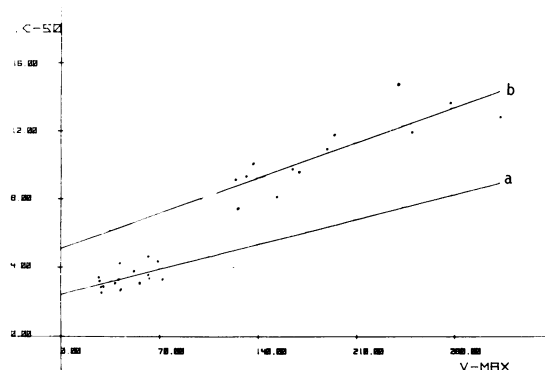
FIGURE 21. Dependence of IC_{50} of metyrapone on V_{max} for ethylmorphine-N-demethylation in (a) 18-day-old and (b) 60-day-old rats.
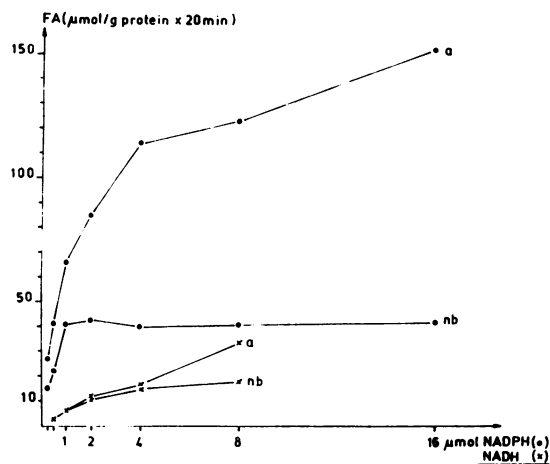

FIGURE 22. Influence of (●) NADPH and (×) NADH alone on N-demethylation of ethylmorphine by liver homogenate of newborn (nb) or adult (a) male rats.

P-450 reduced during this time, the rate of reduction at zero time was calculated. We obtained the following results. The half-life of cytochrome P-450 reduction is the same in all age groups and is slightly reduced after phenobarbital pretreatment. The same holds true for the rate of cytochrome P-450 reduction at zero time; there were no age differneces, but increase by phenobarbital pretreatment. Ethylmorphine or hexobarbital have no clearcut influence on half-life time or on the initial rate of cytochrome P-450 reduction. In summary, age differences corresponding to developmental changes in drug metabolizing activities are not detectable. So the NADPH cytochrome P-450 reductase seems to be not responsible for the age dependence of drug metabolism.

Second Reduction Step. □ With optimal NADP concentrations. additional NADH provides a remarkable further increase with adult liver homogenate as well as with newborn liver (Fig. 23). In both age groups the activity is nearly doubled.

According to Correia and Mannering (*31*), ethylmorphine N-demethylation can be increased by NADH and further by cyanide, blocking the shunting of electrons from cytochrome b_5 to the microsomal fatty acyl-CoA desaturation system. It seemed possible that in young animals with high synthesizing activity this shunt could compete to a greater degree with cytochrome P-450 than in adult animals. We found an activation by NADH with 9.000 g of liver supernatant of adult rats, which is weaker with 15-day-old rats and is not clearly detectable in newborn rats. This NADH effect cannot be demonstrated, however, when the activity of the fortified 9.000 g liver supernatant with NADP and glucose-6-phosphate is increased by NADPH under optimal concentrations. With an optimal NADPH concentration, further addition of NADH is ineffective. Moreover, we could not confirm the cyanide effects, either in adult or in newborns. From these experiments we conclude that an insufficient supply by NADPH and/or NADH or a high loss of electrons by the shunt from cytochrome b_5 to the microsomal fatty acyl-CoA desaturation system is not the reason for the low cytochrome P-450-dependent hydroxylation in newborn animals (*32*).
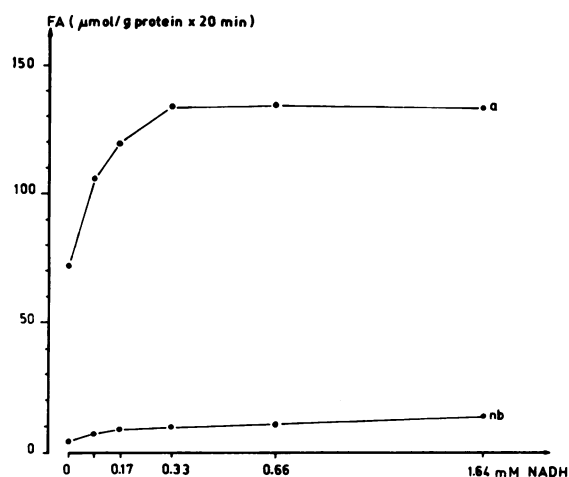

FIGURE 23. Influence of NADH on N-demethylation of ethylmorphine by fortified liver homogenate of newborn (nb) and adult (a) male rats.

The N-demethylation of ethylmorphine by liver microsomes from control and phenobarbital-treated rats of different ages was also investigated, with addition of NADPH in combination with NADH. With NADPH alone (0.6 mmole/l.) we find the typical age curve of V_{max} in control rats as well as after phenobarbital pretreatment (Fig. 24). The higher the activity of N-demethylation of ethylmorphine with NADPH alone, the more it is absolutely enhanced by NADH. The relative increase in ethylmorphine metabolism caused by NADH is equal in all age groups, however, in controls as well as after induction (Fig. 25).

The enhancing effect of NADH is higher at lower NADPH concentrations. In the presence of NADH, the NADPH concentrations necessary to obtain a maximum metabolic rate are lower than without NADH.

From the similarity of the relative effect it is concluded that there are no differences in the introduction of the second electron in all groups of animals. NADH accelerates the reduction/oxidation cycle of cytochrome P-450 to the same extent in all groups, so that more frequent cycles per unit time are possible. The differences in the absolute NADH effect is explained by different
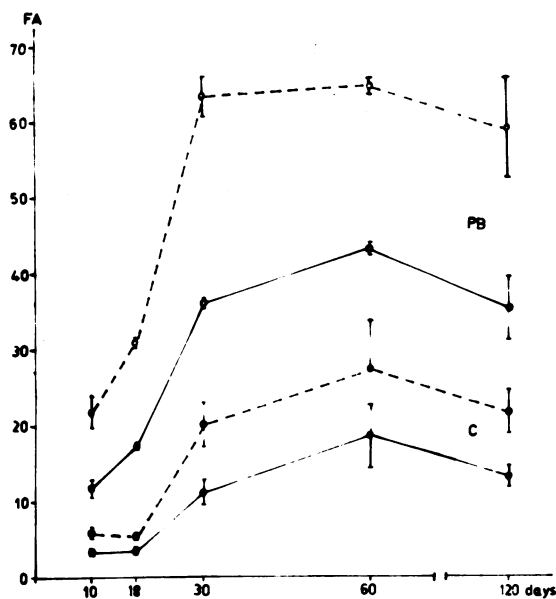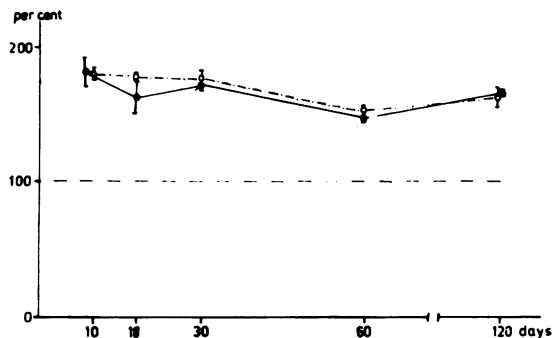

FIGURE 25. Relative change of the rate of N-demethylation of ethylmorphine by NADH: (●) control rats; (O) phenobarbital-pretreated rats, both at optimum NADPH concentrations plus 0.4 mmole/l. NADH; (—) rate without NADH = 100%. Arithmetic means ± S.E.M. are given.

amounts of cytochrome-substrate complex and, consequently, of oxygenated cytochrome-substrate complex.

Conclusions

Low mfO activity (monooxygenase activity) of rat liver during the perinatal period can be observed even under optimal conditions for the in vitro enzyme assay. There are no methodical pitfalls.

The low mfO activity of rat liver during the perinatal period is not due to a lack of NADPH or NADH. It can be demonstrated even under optimal NADPH and NADH supply.

The low mfO activity of rat liver during the perinatal period is not due to an age-dependent NADPH-cyt P-450 reductase activity, or to an age dependent NADH-cyt P-450 reduction.

The low mfO activity of rat liver during the perinatal period is accompained by low ΔA_{max} after addition of a typical type I substrate (hexobarbital) and by a small amount of metyrapone-binding centers. So it can be explained by a smaller percentage or active cyt P-450 in comparison to adult rat liver.

FIGURE 24. Effect of NADH on N-demethylation of ethylmorphine by liver microsomes from (●) control rats (C) and (O) phenobarbital-pretreated rats (PB) of different (ages); (——) optimum NADPH concentration without NADH; (--) optimum NADPH concentrations plus 0.4 mmole/l. NADH. Given are arithmetic means ± S.E.M. FA = nmoles formaldehyde formed per 10 min and 1 mg microsomal protein.

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